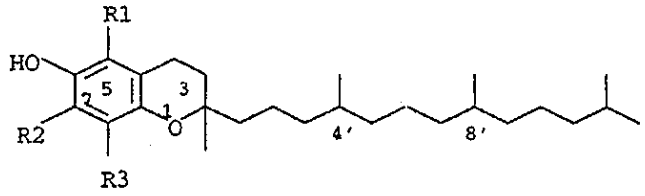
Identification and overexpression of a DNA sequence encoding a 2-methyl-6-phytylhydroquinone methyltransferase in plants

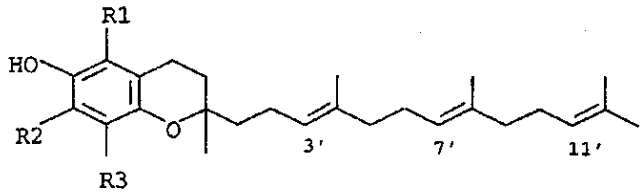
The invention relates to a DNA encoding a polypeptide with 2-methyl-6-phytylhydroquinone methyltransferase activity. Also, the invention relates to the use of DNA sequences encoding a polypeptide with 2-methyl-6-phytylhydroquinone methyltransferase activity for the generation of plants with an elevated tocopherol and tocotrienol content, specifically to the use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 7 or DNA sequences hybridizing herewith or DNA sequences which are homologous to the full sequence or to subsequences, to a method for the generation of plants with an elevated tocopherol and tocotrienol content, and to the resulting plant itself.

The generation of plants with an elevated sugar, enzyme and amino acid content has hitherto been an important objective in plant molecular genetics. The development of plants with an elevated vitamin content, such as, for example, an elevated tocopherol and tocotrienol content, is, however, also of economic interest.

The naturally occurring eight compounds with vitamin E activity are derivatives of 6-chromanol (Ullmann's Encyclopedia of Industrial Chemistry, Vol. A 27 (1996), VCH Verlagsgesellschaft, Chapter 4., 478-488, Vitamin E). The first group (1a-d) is derived from tocopherol, while the second group is composed of tocotrienol derivatives (2a-d):



1a, α-tocopherol: $R^1 = R^2 = R^3 = CH_3$
1b, β-tocopherol [148-03-8]: $R^1 = R^3 = CH_3$, $R^2 = H$
1c, γ-tocopherol [54-28-4]: $R^1 = H$, $R^2 = R^3 = CH_3$
1d, δ-tocopherol [119-13-1]: $R^1 = R^2 = H$, $R^3 = CH_3$



2a, α-tocotrienol [1721-51-3]: $R^1 = R^2 = R^3 = CH_3$
2b, β-tocotrienol [490-23-3]: $R^1 = R^3 = CH_3$, $R^2 = H$
2c, γ-tocotrienol [14101-61-2]: $R^1 = H$, $R^2 = R^3 = CH_3$
2d, δ-tocotrienol [25612-59-3]: $R^1 = R^2 = H$, $R^3 = CH_3$

α-Tocopherol has great economic importance.

The development of crop plants with an elevated tocopherol and tocotrienol content by means of conventional breeding is set a limit.

The genetic engineering approach of isolating essential biosynthesis genes which encode, for example, tocopherol synthesis performance and introducing them into crop plants in a directed fashion is a meaningful alternative. Knowledge of the biosynthesis and its regulation, and identification of genes which affect biosynthesis performance, are prerequisites for this method.

Isoprenoids or terpenoids are composed of a variety of classes of lipid-soluble molecules, and they are formed partially or exclusively from C_5-isoprene units. Pure prenyl lipids (for example carotenoids) are composed of C skeletons based exclusively on isoprene units, while mixed prenyl lipids (for example chlorophylls, tocopherols and vitamin K) have an isoprenoid side chain linked to an aromatic nucleus.

The biosynthesis of prenyl lipids starts with 3 x acetyl-CoA units which are converted into the starting isoprene unit (C_5), namely isopentenyl pyrophosphate (IPP), via ß-hydroxymethylglutaryl-CoA (HMG-CoA) and mevalonate. Recent *in vivo* C^{13} feeding experiments have demonstrated that the IPP formation pathway in various eubacteria, green algae and plant chloroplasts is mevalonate-independent. In this pathway, hydroxyethylthiamine, which is formed by decarboxylation of pyruvate, and glycerolaldehyde-3-phosphate (3-GAP) are first converted into 1-deoxy-D-xylulose-5-phosphate in a "transketolase" reaction mediated by 1-deoxy-D-xylulose-5-phosphate synthase (Lange et al., 1998; Schwender et al., 1997; Arigoni et al., 1997; Lichtenthaler et al., 1997; Sprenger et al., 1997). In an intramolecular rearrangement reaction, this 1-deoxy-D-xylulose-5-phosphate is then converted into 2-C-methyl-D-erythritol-4-phosphate and then into IPP (Arigoni et al., 1997; Zeidler et al., 1998). Biochemical data suggest that the mevalonate pathway operates in the cytosol and leads to the formation of phytosterols. The antibiotic mevinolin, a specific mevalonate formation inhibitor,

only leads to sterol biosynthesis inhibition in the cytoplasma, while prenyl lipid formation in the plastids remains unaffected (Bach and Lichtenthaler, 1993). In contrast, the mevalonate-independent pathway is located in the plastids and leads predominantly to the formation of carotenoids and plastid prenyl lipids (Schwender et al., 1997; Arigoni et al., 1997).

IPP is in equilibrium with its isomer, dimethylallyl pyrophosphate (DMAPP). Condensation of IPP with DMAPP head to tail results in the monoterpene (C_{10}) geranyl pyrophosphate (GPP). Addition of further IPP units results in the sesquiterpene (C_{15}) farnesyl pyrophosphate (FPP), and the diterpene (C_{20}) geranylgeranyl pyrophosphate (GGPP). Bonding between two GGPP molecules results in the formation of the C_{40} precursors of carotenoids.

In the case of mixed prenyl lipids, the isoprene side chain, whose length varies, is linked to non-isoprene rings such as, for example, a porphyrine ring in the case of chlorophylls a and b. The chlorophylls and phylloquinones contain a C_{20} phytyl chain, in which only the first isoprene unit contains a double bond. GGPP is converted by geranylgeranyl pyrophosphate oxidoreductase (GGPPOR) to give phytyl pyrophosphate (PPP), the starting material for the subsequent formation of tocopherols.

The ring structures of the mixed prenyl lipids which lead to the formation of vitamins E and K are quinones whose starting metabolites are derived from the shikimate pathway. The aromatic amino acids phenylalanine or tyrosine are converted into hydroxyphenyl pyruvate, which is dioxygenated to give homogentisic acid. Starting from erythrose-4-phosphate and phosphoenol pyruvate (PEP), the chorismate is formed by their condensation to give 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) via the intermediates of the shikimate pathway, 3'-dehydroquinate, 3'-dehydroshikimate, shikimate, shikimate-3-phosphate and 5'-enolpyruvylshikimate-3-phosphate. During this process, the erythrose-4-phosphate is formed in the Calvin cycle and the PEP is provided during glycolysis. The above-described homogentisic acid is subsequently bound to phytyl pyrophosphate (PPP) or geranylgeranyl pyrophosphate to form the precursors of α-tocopherol and α-tocotrienol, namely 2-methyl-6-phytylhydroquinone and 2-methyl-6-geranylgeranyl hydroquinone, respectively. Methylation steps with S-adenosylmethionine as methyl group donor lead first to 2,3-dimethyl-6-phytylquinol, subsequent cyclization leads to γ-tocopherol and further methylation to α-tocopherol (Richter,

Biochemie der Pflanzen [Plant biochemistry], Georg Thieme Verlag Stuttgart, 1996;).

Examples which demonstrate that manipulation of an enzyme may directionally affect metabolite flow can be found in the literature. A direct effect on the quantities of carotenoids in these transgenic tomato plants was measured in experiments on an altered expression of phytoene synthase, which links two GGPP molecules to give 15-cis-phytoene (Fray and Grierson, Plant Mol. Biol. 22(4) (1993), 589-602; Fray et al., Plant J. 8 (1995), 693-701). As expected, transgenic tobacco plants which have reduced quantities of phenylalanine-ammonium lyase show reduced quantities of phenylpropanoid. The enzyme phenylalanine-ammonium lyase catalyzes the degradation of phenylalanine and thus withdraws it from phenylpropanoid biosynthesis (Bate et al., Proc. Natl. Acad. Sci USA 91 (16) (1994): 7608-7612; Howles et al., Plant Physiol. 112 (1996), 1617-1624;).

Little is known to date on increasing the metabolite flow for elevating the tocopherol and tocotrienol contents in plants by overexpression of individual biosynthesis genes. Only WO 97/27285 describes a modification of the tocopherol content by stronger expression or down-regulation of the enzyme p-hydroxyphenyl pyruvate dioxygenase (HPPD). WO 99/04622 describes a gene sequence encoding a γ-tocopherol methyltransferase from a photosynthetically active organism. WO 99/23231 demonstrates that the expression of a geranylgeranyl reductase in transgenic plants results in an increased tocopherol biosynthesis.

It is an object of the present invention to develop a transgenic plant with an elevated tocopherol and tocotrienol content.

We have found that this object is achieved by overexpressing a 2-methyl-6-phytylhydroquinone methyltransferase gene in plants.

To this end, the activity of 2-methyl-6-phytylhydroquinone methyltransferase (MPMT) was increased by overexpressing the Synechocystis spec. PCC6803 MPMT gene in transgenic plants. This may be achieved in principle by the expression of homologous or heterologous MPMT genes.

Example 2 describes for the first time the cloning of an MPMT DNA sequence (SEQ ID No. 1) from Synechocystis spec. PCC6803. To ensure localization in the plastids, a transit signal sequence is arranged upstream of the Synechocystis MPMT nucleotide sequence (Fig. 3, Fig. 4). Another suitable expression cassette is a DNA sequence encoding an MPMT gene which hybridizes with SEQ ID No. 1

or which is homologous to the full sequence or to subsequences and which is derived from other organisms or plants.

2,3-Dimethyl-6-phytylhydroquinone, of which larger quantities are now available owing to the additional expression of the MPMT gene, is reacted further toward tocopherols and tocotrienol (Figure 1).

The transgenic plants are generated by transforming the plants with a construct comprising the MPMT gene. Model plants employed for the production of tocopherols and tocotrienols were Arabidopsis thaliana, Brassica napus and Nicotiana tabacum.

Measurements on MPMT Synechocystis knock-out mutants showed a drastic decrease regarding the tocopherol and tocotrienol contents. This confirms the direct effect of plastid plant MPMT on tocopherol and tocotrienol synthesis.

The invention relates to the use of a Synechocystis spec. PCC6803 DNA sequence SEQ ID No. 1 which encodes an MPMT or its functional equivalents for the generation of a plant with an elevated tocopherol and tocotrienol content. The nucleic acid sequence may be, for example, a DNA or cDNA sequence. Encoding sequences which are suitable for insertion into an expression cassette are, for example, those which encode an MPMT and which allow the host to overproduce tocopherols and tocotrienols.

The expression cassettes also comprise regulatory nucleic acid sequences which govern the expression of the encoding sequence in the host cell. In a preferred embodiment, an expression cassette comprises a promoter upstream, i.e. on the 5'-end of the encoding sequence, and a polyadenylation signal downstream, i.e. on the 3'-end, and, if appropriate, further regulatory elements which are linked operatively with the sequence in between which encodes the MPMT gene. Operative linkage is to be understood as meaning the sequential arrangement of promoter, encoding sequence, terminator and, if appropriate, further regulatory elements in such a way that each of the regulatory elements can fulfill its function as intended when the encoding sequence is expressed. The sequences preferred for operative linkage, but not restricted thereto, are targeting sequences for guaranteeing subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmatic reticulum (ER), in the nucleus, in elaioplasts or in other compartments, and translation enhancers such as the tobacco mosaic virus 5'-leader sequence (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

As an example, the plant expression cassette can be incorporated into a derivative of the transformation vector pBin-19 with 35S promoter (Bevan, M., Nucleic Acids Research 12 (1984): 8711-8721). Figure 4 shows a derivative of the transformation vector pBin-19 with the seed-specific legumin B4 promoter.

A suitable promoter of the expression cassette is, in principle, any promoter which is capable of governing the expression of foreign genes in plants. In particular, a plant promoter or a promoter derived from a plant virus is preferably used. Particularly preferred is the CaMV 35S promoter from cauliflower mosaic virus (Franck et al., Cell 21 (1980), 285 - 294). As is known, this promoter contains various recognition sequences for transcriptional effectors which in their totality lead to permanent and constitutive expression of the introduced gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

The expression cassette may also comprise a chemically inducible promoter which allows expression of the exogenous MPMT gene in the plant to be governed at a particular point in time. Examples of such promoters which can be used are, inter alia, the PRP1 promoter (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), a salicylic-acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP-A 388186), a tetracyclin-inducible promoter (Gatz et al., (1992) Plant J. 2, 397-404), an abscisic-acid-inducible promoter (EP-A 335528) or an ethanol- or cyclohexanone-inducible promoter (WO 93/21334).

Furthermore, particularly preferred promoters are those which ensure expression in tissues or parts of the plant in which, for example, the biosynthesis of tocopherol or its precursors takes place. Promoters which ensure leaf-specific expression should be mentioned in particular. Promoters which should be mentioned are the potato cytosolic FBPase or the potato ST-LSI promoter (Stockhaus et al., EMBO J. 8 (1989), 2445 - 245).

A foreign protein was expressed stably in the seeds of transgenic tobacco plants to an extent of 0.67% of the total soluble seed protein with the aid of a seed-specific promoter (Fiedler and Conrad, Bio/Technology 10 (1995), 1090-1094). The expression cassette can therefore contain, for example, a seed-specific promoter (preferably the phaseolin promoter (US 5504200), the USP promoter (Baumlein, H. et al., Mol. Gen. Genet. (1991) 225 (3), 459 - 467) or the LEB4 promoter (Fiedler and Conrad, 1995)), the LEB4 signal peptide, the gene to be expressed and an ER retention signal.

An expression cassette is generated by fusing a suitable promoter with a suitable MPMT DNA sequence and, preferably, a DNA which is inserted between promoter and MPMT DNA sequence and which encodes a chloroplast-specific transit peptide, and with a polyadenylation signal, using customary recombination and cloning techniques as they are described, for example, by T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

Particularly preferred sequences are those which ensure targeting into the plastids.

Other expression cassettes which can be used are those whose DNA sequence encodes an MPMT fusion protein, part of the fusion protein being a transit peptide which governs translocation of the polypeptide. Chloroplast-specific transit peptides which are cleaved off enzymatically from the MPMT residue after translocation of the MPMT gene into the chloroplasts are preferred. Particularly preferred is the transit peptide derived from plastid Nicotiana tabacum transketolase or another transit peptide (for example the transit peptide of the Rubisco small subunit or of ferredoxin NADP oxidoreductase) or its functional equivalent.

Especially preferred are DNA sequences of three cassettes of the plastid transit peptide of tobacco plastid transketolase in three reading frames as KpnI/BamHI fragments with an ATG codon in the NcoI cleavage site:

pTP09

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGA
TCC_BamHI

pTP10

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG

TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGCTG
GATCC_BamHI

pTP11

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGG
ATCC_BamHI

The inserted nucleotide sequence encoding an MPMT can be prepared synthetically, obtained naturally or contain a mixture of synthetic and natural DNA constituents, and may be composed of various heterologous MPMT gene segments of a variety of organisms. In general, synthetic nucleotide sequences are produced which are equipped with codons which are preferred by plants. These codons which are preferred by plants can be determined from codons with the highest protein frequency expressed in the majority of interesting plant species. When preparing an expression cassette, a variety of DNA fragments may be manipulated in order to obtain a nucleotide sequence which expediently reads in the correct direction and which is equipped with a correct reading frame. Adaptors or linkers may be added to the fragments in order to link the DNA fragments to each other.

The promoter and terminator regions may expediently be provided, in the direction of transcription, with a linker or polylinker containing one or more restriction sites for insertion of this sequence. As a rule, the linker has 1 to 10, in most cases 1 to 8, preferably 2 to 6, restriction sites. In general, the linker within the regulatory regions has a size less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter may be native, or homologous, or else foreign, or heterologous, to the host plant. The expression cassette comprises, in the 5'-3' direction of transcription, the promoter, a DNA sequence encoding an MPMT gene, and a region for transcriptional termination. Various termination regions may be exchanged for each other as desired.

Manipulations which provide suitable restriction cleavage sites or which eliminate the excess DNA or restriction cleavage sites may also be employed. *In vitro* mutagenesis, primer repair, restriction or ligation may be used in cases where insertions, deletions or substitutions such as, for example, transitions and transversions are suitable. Complementary ends of the fragments may be provided for ligation in the case of suitable

manipulations such as, for example, restriction, chewing back or filling in overhangs for blunt ends.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which correspond essentially to Agrobacterium tumefaciens T-DNA-polyadenylation signals, in particular those of gene 3 of the T-DNA (octopine synthase) of the Ti-plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984), 835 et seq.), or functional equivalents.

The fused expression cassette which encodes an MPMT gene is preferably cloned into a vector, for example pBin19, which is suitable for transforming Agrobacterium tumefaciens. Agrobacteria transformed with such a vector can then be used in a known manner for transforming plants, in particular crop plants, such as, for example, tobacco plants, for example by bathing wounded leaves or leaf sections in an agrobacterial suspension and subsequently growing them in suitable media. The transformation of plants by agrobacteria is known, inter alia, from F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press, 1993, pp. 15 - 38. Transgenic plants which comprise, integrated into the expression cassette, a gene for expressing an MPMT gene can be regenerated in a known manner from the transformed cells of the wounded leaves or leaf sections.

To transform a host plant with a DNA encoding an MPMT, an expression cassette is inserted into a recombinant vector whose vector DNA comprises additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described, inter alia, in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), chapter 6/7, (1993), pp. 71 - 119.

Using the above-cited recombination and cloning techniques, the expression cassettes can be cloned into suitable vectors which allow their multiplication, for example in E. coli. Suitable cloning vectors are, inter alia, pBR332, pUC series, M13mp series and pACYC184. Especially suitable are binary vectors which are capable of replication in E. coli and in agrobacteria.

The invention furthermore relates to the use of an expression cassette comprising a DNA sequence SEQ ID No. 1 or a DNA sequence hybridizing herewith for transforming plants, plant cells, plant tissues or parts of plants. The preferred object of the use is an elevated tocopherol and tocotrienol content of the plant.

Depending on the choice of promoter, expression may take place specifically in the leaves, in the seeds, the petals or in other parts of the plant. Such transgenic plants, their propagation material and the cells, tissues or parts of such plants are a further subject of the present invention.

In addition, the expression cassette may also be employed for transforming bacteria, cyanobacteria, yeasts, filamentous fungi and algae for the purpose of increasing the tocopherol and tocotrienol content.

The transfer of foreign genes into the genome of a plant is termed transformation. It exploits the above-described methods of transforming and regenerating plants from plant tissues or plant cells for transient or stable transformation. Suitable methods are protoplast transformation by polyethylene-glycol-induced DNA uptake, the biolistic method using the gene gun - the so-called particle bombardment method, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and agrobacterium-mediated gene transfer. The abovementioned methods are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993), 128 - 143, and in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205 - 225. The construct to be expressed is preferably cloned into a vector which is suitable for the transformation of Agrobacterium tumefaciens, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711).

Agrobacteria transformed with an expression cassette can equally be used in a known manner for transforming plants, in particular crop plants such as cereals, corn, oats, soya, rice, cotton, sugar beet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and grapevine species, for example by bathing wounded leaves or leaf sections in an agrobacterial suspension and subsequently growing them in suitable media.

Functionally equivalent sequences which encode an MPMT gene are those sequences which still have the desired functions, despite a differing nucleotide sequence. Functional equivalents thus encompass naturally occurring variants of the sequences described herein, and synthetic nucleotide sequences, for example those obtained by chemical synthesis and adapted to suit the codon usage of a plant.

Functional equivalents are also to be understood as meaning, in particular, natural or artificial mutations of an originally isolated sequence encoding an MPMT which continue to show the desired function. Mutations encompass substitutions, additions, deletions, exchanges or insertions of one or more nucleotide residues. Thus, the present invention also encompasses, for example, those nucleotide sequences which are obtained by modifying the MPMT nucleotide sequence. The purpose of such a modification may be, for example, the further limitation of the encoding sequence contained therein or else, for example, the insertion of further restriction enzyme cleavage sites.

Example 8 describes a deletion clone of the MPMT gene, see SEQ ID No. 7.

Functional equivalents are also those variants whose function is attenuated or increased compared with the starting gene, or gene fragment.

Also suitable are artificial DNA sequences as long as they mediate the desired characteristic, for example an elevated tocopherol content in the plant, by overexpression of an MPMT gene in crop plants, as described above. Such artificial DNA sequences can be identified, for example, by backtranslation of proteins with MPMT activity which have been constructed by means of molecular modeling, or else by *in vitro* selection. Especially suitable are encoding DNA sequences which have been obtained by backtranslating a polypeptide sequence in accordance with the host-plant-specific codon usage. An expert skilled in the art of plant genetic engineering methods will readily be able to identify the specific codon usage by computer evaluations of other known genes of the plant to be transformed.

Further suitable equivalent nucleic acid sequences which should be mentioned are sequences which encode fusion proteins, an MPMT polypeptide or a functionally equivalent portion thereof being a constituent of the fusion protein. The second part of the fusion protein may be, for example, another enzymatically active polypeptide, or an antigenic polypeptide sequence with the aid of which detection of MPMT expression is possible (for example myc-tag or his-tag). However, it is preferably a regulatory protein sequence such as, for example, a transit peptide which leads the MPMT protein to the plastids.

An elevated tocopherol and tocotrienol content is to be understood as meaning for the purposes of the present invention the artificially acquired ability to increase biosynthetic

performance regarding these compounds by functional overexpression of an MPMT gene SEQ ID No. 1 or SEQ ID No. 7 in the plant in comparison with the non-genetically modified plant for at least one plant generation.

Both the tocopherol content and the tocotrienol content can be increased. It is preferred to increase the tocopherol content. However, under certain conditions, it is also possible preferentially to increase the tocotrienol content.

The tocopherol biosynthesis site, for example, is inter alia the leaf tissue, so that leaf-specific expression of the MPMT gene is meaningful. However, it is obvious that tocopherol biosynthesis need not be limited to the leaf tissue but may also take place in a tissue-specific fashion in all of the other remaining parts of the plant, for example in fatty seeds.

The constitutive expression of the exogenous MPMT gene is also advantageous. On the other hand, inducible expression may also be desirable.

The expression efficacy of the transgenically expressed MPMT gene can be determined, for example, *in vitro* by shoot meristem propagation. In addition, altered expression of the MPMT gene with regard to type and level, and its effect on tocopherol biosynthesis performance may be tested on test plants in greenhouse experiments.

The invention furthermore relates to transgenic plants, transformed with an expression cassette comprising the sequence SEQ ID No. 1 or SEQ ID No. 7 or a DNA sequence hybridizing herewith or a DNA sequence which is homologous to the full sequence or to subsequences, and to transgenic cells, tissues, parts and propagation material of such plants. Especially preferred are transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soya, rice, cotton, sugar beet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, tagetes, lettuce and the various tree, nut and grapevine species.

Plants for the purposes of the invention are mono- and dicotyledonous plants.

The invention furthermore relates to photosynthetically active organisms transformed with an expression cassette containing the sequence SEQ ID No. 1 or SEQ ID No. 7 or a DNA sequence hybridizing herewith or a DNA sequence which is homologous to the

full sequence or to subsequences. Examples of photosynthetically active organisms are, besides the plants, cyanobacteria, mosses and algae.

Since this biosynthetic pathway is a metabolic pathway which is exclusively located in the plastids, it offers optimal target enzymes for the development of inhibitors. Since, according to current knowledge, no enzyme which is identical or similar to Synechocystis MPMT is present in human and animal organisms, it can be assumed that inhibitors should have a very specific effect on plants.

As already mentioned, MPMT is a potential target for herbicides. To find efficient MPMT inhibitors, it is necessary to provide suitable test systems with which inhibitor-enzyme binding studies can be carried out. To this end, for example, the complete cDNA sequence of the Synechocystis MPMT is cloned into an expression vector (pQE, Qiagen) and overexpressed in *E. coli*.

The MPMT protein which is expressed with the aid of the expression cassette according to the invention is particularly suitable for finding MPMT-specific inhibitors.

To this end, MPMT can be employed, for example, in an enzyme test in which the MPMT activity is determined in the presence and absence of the active ingredient to be tested. A qualitative and quantitative statement on the inhibitory behavior of the active ingredient to be tested can be made by comparing the two activity determinations.

The test system according to the invention allows a large number of chemical compounds to be checked rapidly and simply for herbicidal properties. The method allows reproducibly and specifically to select, from a large number of substances, those with high potency in order subsequently to carry out, with these substances, further, in-depth tests with which the skilled worker is familiar.

The invention furthermore relates to herbicides which can be identified with the above-described test system.

Overexpression of the MPMT-encoding gene sequence SEQ ID No. 1 or SEQ ID No. 7 in a plant allows an improved resistance to MPMT inhibitors to be achieved. Transgenic plants generated thus are also a subject of the invention.

The MPMT protein prepared using the DNA sequence SEQ ID No. 1 or SEQ ID No. 7 is also suitable for carrying out biotransformations for providing substantial amounts of 2,3-dimethyl-6-phytylhydroquinone. To do this, 2-methyl-6-phytylhydroquinone is converted in the presence of the enzyme MPMT and the co-substrate S-adenosyl-L-methionine to give 2,3-dimethyl-6-phytylhydroquinone. In principle, the biotransformation can be carried out on entire cells which express the enzyme MPMT, or on cell extracts of these cells, or else on purified or ultrapure MPMT in the presence of S-adenosyl-L-methionine.

The invention furthermore relates to:

- Methods for the transformation of a plant, which comprise introducing, into a plant cell or protoplasts of plants, expression cassettes containing a DNA sequence SEQ ID No. 1 or SEQ ID No. 7 or a DNA sequence hybridizing herewith or a DNA sequence which is homologous to the full sequence or to subsequences, and regenerating these to give entire plants.

- The use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 7 or a DNA sequence hybridizing herewith for the generation of plants with an elevated tocopherol and tocotrienol content by expressing, in plants, an MPMT DNA sequence.

The invention is illustrated by the examples which follow, but not limited thereto.

Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using a laser fluorescence DNA sequencer by Licor (sold by MWG Biotech, Ebersbach) using the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463 - 5467).

Example 1

Identification of a Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase

The Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase was cloned and identified as follows:

Using a sequence motif conserved in S-adenosyl-L-methionine methyltransferases which is responsible for binding S-adenosyl-L-methionine (SAM) (C.P. Joshi and V.L. Chiang. PMB.

37 (1998): 663-374), a Synechocystis spec. PCC6803 genomic DNA database was screened (Kaneko et al., DNA Res. 34 (1996): 109-136). The hypothetical proteins identified during this screening which contained the SAM binding motif were compared with the primary sequences of the Synechocystis spec. PCC6803 γ-tocopherol methyltransferase (termed slr0089) and the Arabidopsis thaliana γ-tocopherol methyltransferase (David Shintani and Dean DellaPenna. Sience. 282 (1998): 2098-2100).

It was possible to identify a hypothetical protein (termed sll0418 SEQ.-ID No. 2) whose amino acid sequence corresponded to a low degree with the γ-tocopherol methyl transferases from Synechocystis spec. PCC6803 and Arabidopsis thaliana (36% and 28% identity, respectively).

Further studies of the primary sequence of the hypothetical protein sll0418 confirmed the existence of a putative prokaryotic signal sequence within the first 20 amino acids (PSIGNAL, PC/GENE™ IntelliGenetics, Inc (1991). Such a sequence was also identified in Synechocystis spec. PCC6803 γ-tocopherol methyltransferase (slr0089) (D. Shintani and D. DellaPenna., Sience. 282 (1998): 2098-2100) and suggests the identical localization of the two proteins.

The predicted molecular weight of the unprocessed protein is 34.9 kDa and is thus within a range which had also been determined for the Synechocystis spec. PCC6803 γ-tocopherol methyltransferase (David Shintani and Dean DellaPenna, Sience. 282 (1998): 2098-2100) and for the γ-tocopherol methyltransferase purified from bell peppers (d'Harlingue and Camara, Plastid enzymes of terpenoid biosynthesis: Purification of γ-Tocopherol Methyltransferase from Capsicum Chromoplasts. Journal of Biological Chemistry, Vol. 269 (1985), No.28, 15200-15203).

Taking into consideration the facts, we concluded that the hypothetical protein sll0418 might be a tocopherol methyltransferase.

Example 2

Amplification and cloning of the Synechocystis spec. PCC 6803 2-methyl-6-phytylhydroquinone methyltransferase

The DNA encoding the ORF (open reading frame) sll0418 was amplified by means of polymerase chain reaction (PCR) from Synechocystis spec. PCC6803 following the method of Crispin A. Howitt (BioTechniques 21, July 1996:32-34) using a sense-specific

primer (sll04185' SEQ ID No. 5) and an antisense-specific primer (sll04183' SEQ ID No. 6).

The PCR conditions were as follows:

The PCR was carried out in a 50 µl reaction batch which contained:

- 5 µl of a Synechocystis spec. PCC6803 cell suspension
- 0.2 mM dATP, dTTP, dGTP, dCTP
- 1.5 mM $Mg(OAc)_2$
- 5 µg bovine serum albumin
- 40 pmol sll04185'
- 40 pmol sll04183'
- 15 µl 3.3 × rTth DNA polymerase XL buffer (PE Applied Biosystems)
- 5U rTth DNA polymerase XL (PE Applied Biosystems)

The PCR was carried out under the following cycle conditions:

Step 1: 5 minutes at 94°C (denaturing)
Step 2: 3 seconds at 94°C
Step 3: 2 minutes at 58°C (annealing)
Step 4: 2 minutes at 72°C (elongation)
40 repetitions of steps 2-4
Step 5: 10 minutes at 72°C (post-elongation)
Step 6: 4°C (waiting loop)

The amplicon was cloned into the PCR cloning vector pGEM-T (Promega) using standard methods. The identity of the amplicon generated was confirmed by sequencing using the M13F (-40) primer.

Example 3

Generation of an sll0418 knock-out mutant

A DNA construct to generate a deletion mutant of the ORF sll0418 in Synechocystis spec. PCC6803 was generated using standard cloning techniques.

The vector pGEM-T/sll0418 was digested using the restriction enzyme BalI. The presence of two BalI cleavage sites within the sll0418 sequence (position bp 109 and bp 202, respectively) resulted in the loss of a 93-bp internal fragment. The aminoglycoside-3'-phosphotransferase of the transposon Tn903 was cloned into the BalI cleavage sites of the sll0418 ORF. To this

end, the Tn903 was isolated as an EcoRI fragment from the vector pUC4k (Vieira, J and Messing, J, Gene: 19 (1982), 259-268), the overhangs of the restriction digest were made blunt-ended by standard methods and ligated into the BalI-cut vector pGEM-T/sll0418. The ligation batch was used for the transformation of *E. coli* Xl1 blue cells. Transformants were selected by using kanamycin and ampicillin. A recombinant plasmid (pGEM-T/sll0418::tn903) was isolated and employed in the transformation of Synechocystis spec. PCC6803 following the method of Williams (Methods Enzymol. 167 (1987), 776-778).

Synechocystis spec. PCC6803 transformants were selected on kanamycin-containing (kan) solid BG-11 medium (Castenholz, Methods in Enzymology (1988), 68-93) at 28°C and 30 µmol photons $\times (m^2 \times s)^{-1}$. After five selection cycles (passages of single colonies onto fresh BG-11 kan medium), four independent knock-out mutants were generated.

The complete loss of the sll0418 endogene or the exchange for the recombinant sll0418::tn903 DNA was confirmed by PCR analyses.

Example 4

Comparison of the tocopherol production in Synechocystis spec. PCC6803 wild-type cells and the generated knock-out mutants of ORF sll0418

The cells of the four independent Synechocystis spec. PCC68033 knock-out mutants of ORF sll0418 which were cultured on the BG-11 kan agar medium and untransformed wild-type cells were used to inoculate liquid cultures. These cultures were grown for approximately 3 days at 28°C and 30 µmol photons $\times (m^2 \times s)^{-1}$ (30 µE). After determining the OD_{730} of the individual cultures, the OD_{730} of all cultures was synchronized by suitable dilutions with BG-11 (wild types) or BG-11 kan (mutants). These cell-density-synchronized cultures were used to inoculate three cultures per mutant and the wild-type controls. Thus, the biochemical analyses were carried out using in each case three independently grown cultures of a mutant and the corresponding wild types. The cultures were grown to an optical density of OD_{730}=0.3. The cell culture medium was removed by two centrifugation steps at 14,000 rpm in an Eppendorf bench centrifuge. The cells were subsequently disrupted by four incubations for 15 minutes in an Eppendorf shaker at 30°C, 1000 rpm, in 100% methanol, and the supernatants obtained in each

case were combined. Further incubation steps resulted in no further release of tocopherols or tocotrienols.

To avoid oxidation, the resulting extracts were analyzed directly after extraction with the aid of a Waters Allience 2690 HPLC system. Tocopherols and tocotrienols were separated over a reverse phase column (ProntoSil 200-3-C30, Bischoff) using a mobile phase of 100% methanol, and identified with reference to standards (Merck). The detection system used was the fluorescence of the substances (excitation 295 nm, emission 320 nm), which was detected with the aid of a Jasco fluorescence detector FP 920.

In the Synechocystis spec. PCC6803 knock-out mutants of ORF sll0418, no tocopherols or tocotrienols were found. However, tocopherols and tocotrienols were measured in the Synechocystis spec. PCC6803 wild-type cells.

The loss of the ability to produce tocopherols and tocotrienols within the knock-out mutants of ORF sll0418 in comparison with the Synechocystis spec. PCC6803 wild-type cells demonstrates that the gene sll0418 encodes a 2-methyl-6-phytylhydroquinone methyltransferase.

Example 5

Functional characterization of the Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase by heterologous expression in E. coli

The hypothetic Synechocystis spec. PCC6803 protein sll0418 was identified by functional expression in *E. coli* as 2-methyl-6-phytylhydroquinone methyltransferase.

The gene sll0418 which had been amplified from Synechocystis spec. PCC6803 was subcloned into the expression vector pQE-30 (Qiagen) in the correct reading frame. The primers sll104185' and sll04183' (SEQ ID No. 5 and 6, respectively) which had been used for amplifying the ORF sll0418 from Synechocystis spec. PCC6803, were constructed in such a way that BamHI restriction cleavage sites were added to the 5' end and the 3' end of the amplicon; see SEQ ID No. 3. Using these flanking BamHI restriction cleavage sites, the sll0418 fragment was isolated from the recombinant plasmid pGEM-T/sll0418 and ligated into a BamHI-cut pQE-30 using standard methods. The ligation batch was used for the transformation of M15 *E. coli* cells, and kanamycin- and ampicillin-resistant transformants were analyzed. The kanamycin resistance is mediated by the pREP-4 plasmid, which is contained

in the M15 cells. A recombinant plasmid (pQE-30/sll0418) which carried the sll0418 fragment in the correct orientation was isolated. Identity and orientation of the insert were confirmed by sequencing.

The recombinant plasmid pQE-30/sll0418 was used for the transformation of M15 *E. coli* cells in order to generate recombinant sll0418 protein. Using a colony which originated from the transformation, an overnight culture in Luria broth medium supplemented with 200 µg/ml ampicillin (Amp) and 50 µg/ml kanamycin (Kan) was inoculated. Starting from this culture, a 100 ml Luria broth culture (Amp/Kan) was inoculated the morning thereafter. This culture was incubated at 28°C on a shaker incubator until an OD_{600} of 0.35-0.4 was reached. Then, production of the recombinant protein was induced by adding 0.4 mM isopropyl-ß-D-thiogalactopyranoside (IPTG). The culture was shaken for a further 3 hours at 28°C and the cells were subsequently pelleted by centrifugation at 8000 g.

The pellet was resuspended in 600 µl lysis buffer (approx. 1-1.5 ml/g pellet moist weight, 10 mM HEPES KOH pH 7.8, 5 mM dithiothreitol (DTT), 0.24 M sorbitol). Then, PMSF (phenyl methylsulfonate) was added to a final concentration of 0.15 mM and the batch was placed on ice for 10 minutes. The cells were disrupted by a 10-second ultrasonic pulse using an ultrasonic processor. After addition of Triton X100 (final concentration 0.1%), the cell suspension was incubated on ice for 30 minutes. The batch was subsequently centrifuged for 30 minutes at 25,000 x g and the supernatant was employed in the assay.

The 2-methyl-6-phytylhydroquinone methyltransferase activity was determined by detecting the radiolabeled reaction product 2,3-dimethyl-6-phytylhydroquinone.

To this end, 135 µl of the enzyme (approx. 300-600 µg) together with 20 µl of substrate (2-methyl-6-phytylhydroquinone) and 15 µl (0.46 mM SAM ^{14}C) methyl group donor were incubated for 4 hours in the dark at 25°C in the following reaction buffer: 200 µl (125 mM) tricine-NaOH pH 7.6, 100 µl (1.25 mM) sorbitol, 10 µl (50 mM) $MgCl_2$ and 20 µl (250 mM) ascorbate.

The reaction was quenched by adding 750 µl of chloroform/methanol (1:2) + 150 µl of 0.9% NaCl. The mixed batch was centrifuged briefly, and the top phase was discarded. The bottom phase is transferred into a new reaction vessel and evaporated under nitrogen. The residues were taken up in 20 µl of ether and applied to a thin-layer plate in order to separate the substances

by chromatography (solid phase: HPTLC plates: silica gel 60 F_{254} (Merck), liquid phase: toluene). The radiolabeled reaction product is detected by using a phosphoimager.

These experiments confirm that the protein encoded by the Synechocystis spec. PCC6803 gene sll0418 (SEQ ID No.1) is a 2-methyl-6-phytylhydroquinone methyltransferase since it has the enzymatic activity to convert 2-methyl-6-phytylhydroquinone into 2,3-dimethyl-6-phytylhydroquinone.

Figure 2 shows a sequence comparison at amino acid level between the γ-tocopherol methyltransferases from Synechocystis spec. PCC Synechocystis spec. PCC6803 (slr0089) and *A.thaliana* (aratmt) with the 2-methyl-6-phytylhydroquinone methyltransferase (sll0418) from Synechocystis spec. PCC6803. The correspondence with the γ-tocopherol methyltransferases from Synechocystis spec. PCC6803 and Arabisopsis thaliana is 36 and 28% identity, respectively.

Example 6

Substrate specificity of the 2-methyl-6-phytylhydroquinone methyltransferase

Enzymatic studies as carried out in Example 5 confirm that the enzyme MPMT - encoded by the Synechocystis spec. PCC6803 gene sll0418 (SEQ-ID No. 1) - converts 2-methyl-6-phytylhydroquinone in 2,3-dimethyl-6-phytylhydroquinone.

In addition, the enzyme MPMT has a 2-methyl-6-geranyl-geranylhydroquinone methyltransferase activity, while a γ-tocopherol methyltransferase activity could not be detected. This confirms that the enzyme 2-methyl-6-phytylhydroquinone methyltransferase participates in tocotrienol biosynthesis since it converts 2-methyl-6-geranylgeranylhydroquinone into 2,3-dimethyl-6-geranylgeranylhydroquinone. This clearly demonstrates that the enzyme activity of the 2-methyl-6-phytylhydroquinone methyltransferase differs from that of γ-tocopherol methyltransferase.

Example 7

Generation of expression cassettes containing the MPMT gene

Transgenic plants were generated which express the Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase firstly under the control of the constitutive CaMV (cauliflower

mosaic virus) 35S promoter (Franck et al., cell 21 (1980), 285-294) and secondly under the control of the seed-specific promoter of the *Vicia faba* legumin gene (Kafatos et al., Nuc. Acid. Res., 14(6) (1986), 2707-2720). The basis of the plasmid generated for the constitutive expression of the Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase was pBinAR-TkTp-9 (Ralf Badur, PhD thesis, University of Göttingen, 1998). This vector is a derivative of pBinAR (Höfgen and Willmitzer, Plant Sci. 66 (1990), 221-230) and contains the CaMV (cauliflower mosaic virus) 35S promoter (Franck et al., 1980), the termination signal of the octopine synthase gene (Gielen et al., EMBO J. 3 (1984), 835-846) and the DNA sequence encoding the transit peptide of the *Nicotiana tabacum* plastid transketolase (Ralf Badur, PhD thesis, University of Göttingen, 1998). Cloning the Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase into this vector taking into consideration the correct reading frame generates a translational fusion of the 2-methyl-6-phytylhydroquinone methyltransferase with the plastid transit peptide. The transgene is thus transported to the plastids.

To generate this plasmid, the gene sll0418 was isolated from plasmid pGEM-T/sll0418 using the flanking BamHI restriction cleavage sites. This fragment was ligated into a BamHI-cut pBinAR-TkTp-9 using standard methods (see Figure 3). This plasmid (pBinAR-TkTp-9/sll0418) was used for the generation of transgenic Arabidopsis thaliana, Brassica napus and Nicotiana tabacum plants. Fragment A (529 bp) in Figure 3 contains the CaMV 35S promoter (nucleotides 6909 to 7437 of cauliflower mosaic virus), fragment B (245 bp) encodes the transit peptide of the *Nicotiana* tabacum transketolase, fragment C (977 bp) encodes the Synechocystis spec. PCC6803 ORF sll0418, and fragment D (219 bp) encodes the termination signal of the octopine synthase gene.

To generate a plasmid which allows the seed-specific expression of the Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase in plants, the seed-specific promoter of the legumin B4 gene (Kafatos et al., Nuc. Acid. Res., 14(6) (1986), 2707-2720), was used. The 2.7 kb fragment of the legumin B4 gene promoter was isolated from plasmid pCR-Script/lePOCS using the EcoRI cleavage site and the KpnI cleavage site which flank the promoter at the 5′ and at the 3′ end, respectively. Plasmid pBinAR-TkTp-9/sll0418 was also treated with the restriction enzymes EcoRI and KpnI. As a consequence, the CaMV 35S promoter was excised from this plasmid. The promoter of the legumin gene was subsequently cloned into this vector as EcoRI/KpnI fragment, thus generating a plasmid which placed the expression of gene

sll0418 under the control of this seed-specific promoter, see Figure 4. This plasmid (pBinARleP-TkTp-9/sll0418) was used for the generation of transgenic Arabidopsis thaliana, Brassica napus and Nicotiana tabacum plants.

Fragment A (2700 bp) in Figure 4 contains the promoter of the *Vicia faba* legumin B4 gene, fragment B (245 bp) encodes the transit peptide of the *Nicotiana tabacum* transketolase, fragment C (977 bp) encodes the Synechocystis spec. PCC6803 ORF sll0418, and fragment D (219 bp) encodes the termination signal of the octopine synthase gene.

Example 8

Generation of expression cassettes containing a deletion clone of the MPMT gene

Based on a computer analysis, a putative prokaryotic secretion signal was identified in the primary sequence of ORF sll0418. To ensure that this has no adverse effect on the import of the protein into the plastids upon expression in plants, a derivative of the sequence of sll0418 was generated in which the putative secretion signal was deleted (SEQ ID No. 7). This deletion was carried out using PCR technology. By virtue of the primers used for this purpose (sll0418DSp5', SEQ ID No. 9 and sll0418DSp3', SEQ ID No. 10), an EcoRV restriction cleavage site was added onto the 5' end of the sequence and an SalI restriction cleavage site onto the 3' end, thus allowing directed cloning into vector pBinAR-TkTp-9. The resulting plasmid pBinAR-TkTp-9/sll0418ΔSP is described in Figure 5. Fragment A (529 bp) in Figure 5 contains the CaMV 35S promoter (nucleotides 6909 to 7437 of the cauliflower mosaic virus), fragment B (245 bp) encodes the transit peptide of the *Nicotiana tabacum* transketolase, fragment C (930 bp) encodes the Synechocystis spec. PCC6803 ORF sll0418ΔSP, and fragment D (219 bp) encodes the termination signal of the octopine synthase gene.

To generate a plasmid which allows the seed-specific expression of the deletion clone of the Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase in plants, the seed-specific promoter of the legumin B4 gene which has already been described was used again (Kafatos et al., Nuc. Acid. Res., 14(6) (1986), 2707-2720). The 2.7 kb fragment of the legumin B4 gene promoter was isolated from plasmid PCR-Script/lePOCS using the EcoRI and the KpnI cleavage sites which flank the promoter at the 5' end and at the 3' end, respectively. Plasmid pBinAR-TkTp-9/sll0418ΔSP was also treated with the restriction

enzymes EcoRI and KpnI. As a consequence, the CaMV 35S promoter was excised from this plasmid. The promoter of the legumin gene was subsequently cloned into this vector as an EcoRI/KpnI fragment, thus generating a plasmid which placed the expression of the deletion clone of the sll0418 gene under the control of this seed-specific promoter, see Figure 6. Fragment A (2700 bp) in Figure 6 contains the promoter of the Vicia faba legumin B4 gene, fragment B (245 bp) encodes the transit peptide of Nicotiana tabacum transketolase, fragment C (930 bp) encodes the Synechocystis spec. PCC6803 ORF sll0418ΔSP, and fragment D (219 bp) encodes the termination signal of the octopine synthase gene.

This plasmid (pBinARleP-TkTp-9/sll0418ΔSP) was used for the generation of transgenic Arabidopsis thaliana, Brassica napus and Nicotiana tabacum plants.

An increase in the tocopherol and tocotrienol contents was also measured by expressing the DNA sequence SEQ-ID No. 7 in transgenic plants.

Example 9

Generation of transgenic Arabidopsis thaliana plants

Wild-type Arabidopsis thaliana plants (Columbia) were transformed with the Agrobacterium tumefaciens strain (EHA105) using a modified vacuum infiltration method as the basis (Steve Clough and Andrew Bent. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16(6):735-43, 1998; Bechtold, N., Ellis, J. and Pelltier, G., in: Planta Agrobacterium-mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. CRAcad Sci Paris, 1993, 1144(2):204-212). The Agrobacterium tumefaciens cells used had previously been transformed with plasmids pBinAR-TkTp-9/sll0418 or pBinARleP-TkTp-9/sll0418 (Figure 3 and 4).

Seeds of the primary transformants were selected on the basis of antibiotic resistance. Antibiotic-resistant seedlings were planted into soil and used for biochemical analysis as fully developed plants.

Example 10

Generation of transgenic Brassica napus plants

The generation of transgenic oilseed rape plants followed in principle a procedure described by Bade, J.B. and Damm, B. (in Gene Transfer to Plants, Potrykus, I. and Spangenberg, G., eds, Springer Lab Manual, Springer Verlag, 1995, 30-38), which also gives the composition of the media and buffers used.

The transformations were performed with Agrobacterium tumefaciens strain EHA105. Plasmids pBinAR-TkTp-9/s110418 and pBinARleP-TkTp-9/s110418 were used for the transformation. Seeds of Brassica napus var. Westar were surface-sterilized with 70% ethanol (v/v), washed in water for 10 minutes at 55°C, incubated for 20 minutes in 1% strength hypochlorite solution (25% v/v Teepol, 0.1% v/v Tween 20) and washed six times with sterile water for in each case 20 minutes. The seeds were dried on filter paper for three days, and 10-15 seeds were germinated in a glass flask containing 15 ml of germination medium. The roots and apices were removed from several seedlings (approximate size 10 cm), and the hypocotyls which remained were cut into sections of approximate length 6 mm. The approximately 600 explants thus obtained were washed for 30 minutes with 50 ml of basal medium and transferred into a 300 ml flask. After addition of 100 ml of callus induction medium, the cultures were incubated for 24 hours at 100 rpm.

An overnight culture of the agrobacterial strain was established in Luria broth medium supplemented with kanamycin (20 mg/l) at 29°C, and 2 ml of this were incubated in 50 ml of Luria broth medium without kanamycin for 4 hours at 29°C to an OD_{600} of 0.4 - 0.5. After the culture had been pelleted for 25 minutes at 2000 rpm, the cell pellet was resuspended in 25 ml of basal medium. The bacterial concentration in the solution was brought to an OD_{600} of 0.3 by adding more basal medium.

The callus induction medium was removed from the oilseed rape explants using sterile pipettes, 50 ml of agrobacterial suspension were added, the cultures were mixed carefully and incubated for 20 minutes. The agrobacterial suspension was removed, the oilseed rape explants were washed for 1 minute with 50 ml of callus induction medium, and 100 ml of callus induction medium were subsequently added. Cocultivation was performed for 24 hours on an orbital shaker at 100 rpm. Cocultivation was stopped by removing the callus induction medium, and the explants were washed twice with 25 ml of wash medium for 1 minute each

time and twice for 60 minutes with 100 ml of wash medium each time, at 100 rpm. The wash medium together with the explants was transferred into 15 cm Petri dishes, and the medium was removed using sterile pipettes.

For regeneration, batches of 20-30 explants were transferred into 90 mm Petri dishes containing 25 ml of shoot induction medium supplemented with kanamycin. The Petri dishes were sealed with two layers of Leukopor and incubated at 25°C and 2000 lux at photoperiods of 16 hours light/8 hours dark. Every 12 days, the developing calli were transferred to fresh Petri dishes containing shoot induction medium. All further steps for regenerating entire plants were carried out as described by Bade, J.B. and Damm, B. (in Gene Transfer to Plants, Potrykus, I. and Spangenberg, G., eds, Springer Lab Manual, Springer Verlag, 1995, 30-38).

Example 11

Generation of transgenic Nicotiana tabacum plants

10 ml of YEB medium supplemented with antibiotic (5 g/l beef extract, 1 g/l yeast extract, 5 g/l peptone, 5 g/l sucrose and 2 mM $MgSO_4$) were inoculated with a colony of Agrobacterium tumefaciens and the culture was grown overnight at 28°C. The cells were pelleted for 20 minutes at 4°C, 3500 rpm, using a bench centrifuge and then resuspended under sterile conditions in fresh YEB medium without antibiotics. The cell suspension was used for the transformation.

The sterile-grown wild-type plants were obtained by vegetative propagation. To this end, only the tip of the plant was cut off and transferred to fresh 2MS medium in a sterile preserving jar. As regards the rest of the plant, the hairs on the upper side of the leaves were removed and the central veins of the leaves were removed. Using a razor blade, the leaves were cut into sections of approximate size 1 cm^2. The agrobacterial culture was transferred into a small Petri dish (diameter 2 cm). The leaf sections were briefly drawn through this solution and placed with the underside of the leaves on 2MS medium in Petri dishes (diameter 9 cm) in such a way that they touched the medium. After two days in the dark at 25°C, the explants were transferred to plates with callus induction medium and warmed at 28°C in a controlled-environment cabinet. The medium had to be changed every 7-10 days. As soon as calli formed, the explants were transferred into sterile preserving jars on shoot induction medium supplemented with claforan (see above). Organogenesis

started after approximately one month and it was possible to cut off the shoots formed. The shoots were grown on 2MS medium supplemented with claforan and selection marker. As soon as a substantial root ball had developed, it was possible to pot up the plants in seed compost.

Example 12

Characterization of the transgenic plants

To confirm that expression of the Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase increased vitamin E biosynthesis in the transgenic plants, the tocopherol and tocotrienol contents in leaves and seeds of the plants transformed with the constructs pBinARleP-TkTp-9/sll0418 or pBinAR-TkTp-9/sll0418 (Arabidopsis thaliana, Brassica napus and Nicotiana tabacum) were analyzed. To this end, the transgenic plants were grown in the greenhouse, and plants which express the gene encoding the Synechocystis spec. PCC6803 2-methyl-6-phytylhydroquinone methyltransferase were analyzed at Northern level. The tocopherol content and the tocotrienol content in leaves and seeds of these plants were determined. In all cases, the tocopherol or tocotrienol concentration in transgenic plants which additionally express a DNA sequence SEQ ID No. 1 or SEQ ID No. 7, was elevated in comparison with untransformed plants.